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A Strategy for the Isolation of Catalytic Activities from Repertoires of Enzymes Displayed on Phage

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We have aimed at developing a general methodology for the isolation of enzymatic activities from large repertoires of protein displayed on the surface of a filamentous phage. When selecting for protein binders by phage display, phage particles with suitable specificities are physically isolated by affinity capture and amplified by bacterial infection. Selection for catalysis mediated by enzymes displayed on filamentous phage is more difficult, as reaction products (which represent the biochemical memory of the reaction catalysed by the phage particle) diffuse away after the reaction is complete. We reasoned that if we were able to anchor the reaction products on the phage surface, the catalytically active phages could then be physically isolated using specific anti-product affinity reagents.

We achieve the conditional anchoring of reaction substrates and products on phage by displaying enzyme-calmodulin chimeric proteins on filamentous phage as gene III fusions. Such phage particles can be targeted in a stable fashion ($k_{off} < 10^{-4}$ s^{-1}) by chemical derivatives of a calmodulin-binding peptide. The peptide-phage complexes are stable in purification procedures such as capture with magnetic beads and polyethylene glycol precipitation, and can be conditionally dissociated by addition of calcium chelators. Glutathione-*S*-transferase and an endopeptidase were used in model selection experiments to demonstrate that it is possible to isolate catalytic activities from calmodulin-tagged enzymes displayed on filamentous phage, with enrichment factors >50 per round of selection.

Keywords: phage display; calmodulin; calmodulin binding peptides; enzyme selection

Introduction

The development of enzymes which catalyse novel reactions is a daunting biochemical challenge, which promises to be important for the understanding of how enzymes work (Fersht, 1984), for the production of fine chemicals (Whitesides & Wong, 1985; Jackson *et al.*, 1994; Wagner *et al.*, 1995) and for medical applications (Bosslet *et al.*, 1994; Kerr *et al.*, 1995). The isolation of novel useful enzymes should, in principle, be possible, if one could generate large protein repertoires (for example, by combinatorially mutating residues in the active sites of existing enzymes; Reidhaar-Olson & Sauer, 1988) and if a methodology was available, that allows the efficient selection and amplification of the enzymatic activity of interest.

The display of proteins on the surface of filamentous bacteriophage by fusion to a minor coat protein of phage (pIII; Smith, 1985), together with the generation of large macromolecular reper-

Abbreviations used: PMSF, phenylmethylsulfonyl fluoride; CaM, calmodulin; PEG, polyethyleneglycol 6000; KM13, helper phage KM13; VCS-M13, helper phage VCS-M13; submut, subtilisin mutant; TBS, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl; TBSE, TBS, 20 mM EDTA; TBSC, TBS, 1 mM $CaCl_2$; t.u., transforming units; GST, glutathione-*S*-transferase; biotin-HPDP, *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide; BSA, bovine serum albumin; rbs, ribosome binding site; PBS, phosphate-buffered saline.

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toires and use of powerful selection-amplification schemes, has led to the isolation of several classes of useful proteins. For example, high-affinity antibodies have recently been isolated without immunisation (Winter *et al.*, 1994; Vaughan *et al.*, 1996; Pini *et al.*, 1998). In a similar fashion, DNA-binding proteins with altered DNA-binding specificity (Greisman & Pabo, 1997), improved hormones (Lowman & Wells, 1993) and enzyme inhibitors (Roberts *et al.*, 1992) have been isolated. Enzymes have been expressed on the surface of filamentous phage (McCafferty *et al.*, 1991; Corey *et al.*, 1993; Soumillion *et al.*, 1994; Widersten & Mannervik, 1995). One could use phage display technology for the selection of enzyme activity, for example, by challenging protein libraries on phage with suicide inhibitors (Soumillion *et al.*, 1994) or transition state analogues (Widersten & Mannervik, 1995). A more general methodology, however, would feature the physical isolation of the phage enzymes that convert the substrate of the reaction of interest into the desired product. Such a methodology would directly select for catalysis, and would not require knowledge of the structure of the transition state and of the catalytic mechanism.

We propose the display of enzyme-calmodulin fusions to gene III on the surface of filamentous phage as a means of providing the conditional anchoring of reaction substrates and products on phage (Neri *et al.*, 1997). The principle of the methodology is depicted in Figure 1. The substrate of the reaction of interest is chemically conjugated to a recently isolated high-affinity calmodulin-binding peptide ($K_d = 2$ pM; Montigiani *et al.*, 1996). The aim is to keep the substrate of reaction (and eventually the reaction product) non-covalently but tightly linked to the phage particles. We expect that the phage displaying a catalytically active enzyme will convert the substrate into the product efficiently with an intramolecular reaction. Antibodies (or other affinity reagents) against the reaction product will eventually be used for the physical selection of those clones that have catalysed the reaction. As the complex between calmodulin and the peptide derivative is calcium dependent, a mild and selective elution can be achieved by the addition of calcium chelators.

Recently, Pedersen and colleagues have described a method for directed evolution and functional cloning of enzymes (Pedersen *et al.*, 1998) that has similarities to the method depicted in Figure 1 (Neri *et al.*, 1997). In their scheme, the authors attach the reaction substrate covalently to one or more copies of the minor coat protein pIII of the phage, while another copy of pIII is fused to the enzyme of interest. The linker between substrate and pIII is chosen sufficiently long to allow for an intramolecular reaction on phage.

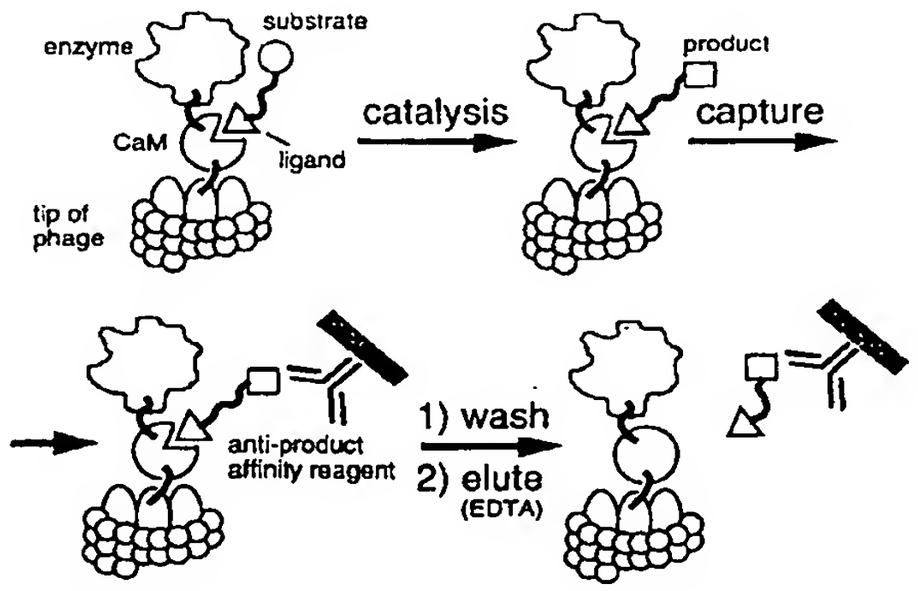


Figure 1. Calmodulin-tagged phage enzyme for the selection of enzymatic activity. High-affinity calmodulin-binding peptides can be used for the non-covalent stable anchoring of reaction substrates and products on the surface of filamentous phage, and for the selection of active phage enzymes with anti-product affinity reagents. The calmodulin/ligand complex can be dissociated by addition of calcium chelators.

Results

Calmodulin fusion proteins can be displayed on filamentous phage

Two phagemid vectors were produced that allow the display of calmodulin-tagged enzymes on filamentous phage (Figure 2). The phagemid vector pDN323 is a derivative of pHEN1 (Hoogenboom *et al.*, 1991) containing a multiple cloning site, the calmodulin gene, an amber codon and the gene III sequentially fused. Phagemid pSD4 is a derivative of pDN323, with a 16 amino acid residue peptide linker between calmodulin and enzyme.

In order to test whether the calmodulin tag of pDN323 was compatible with protein display on filamentous phage, we cloned peptides and folded proteins into pDN323 or pSD4 (Table 1). From these vectors, the corresponding phage particles were produced using standard procedures. In all cases tested, high phage titres were obtained (>10^{10} transforming units (t.u.) per ml in bacterial supernatants), indicating that the calmodulin tag had no deleterious effect on phage production.

The phage preparations were assayed by enzyme-linked immunosorbent assays (ELISA), using suitable affinity reagents (Table 1; Materials and Methods). Figure 3 shows some representative ELISA results obtained with enzymes displayed on calmodulin-tagged phage. Binding to the biotinylated derivative of peptide CAAARWKKAFIAV-SAANRFKKIS with affinity to calmodulin in the picomolar range (Montigiani *et al.*, 1996) was observed in the presence of calcium, but not when the calcium chelator ethylendiaminotetraacetic acid (EDTA) was added. Phage particles not displaying calmodulin did not react in this assay.

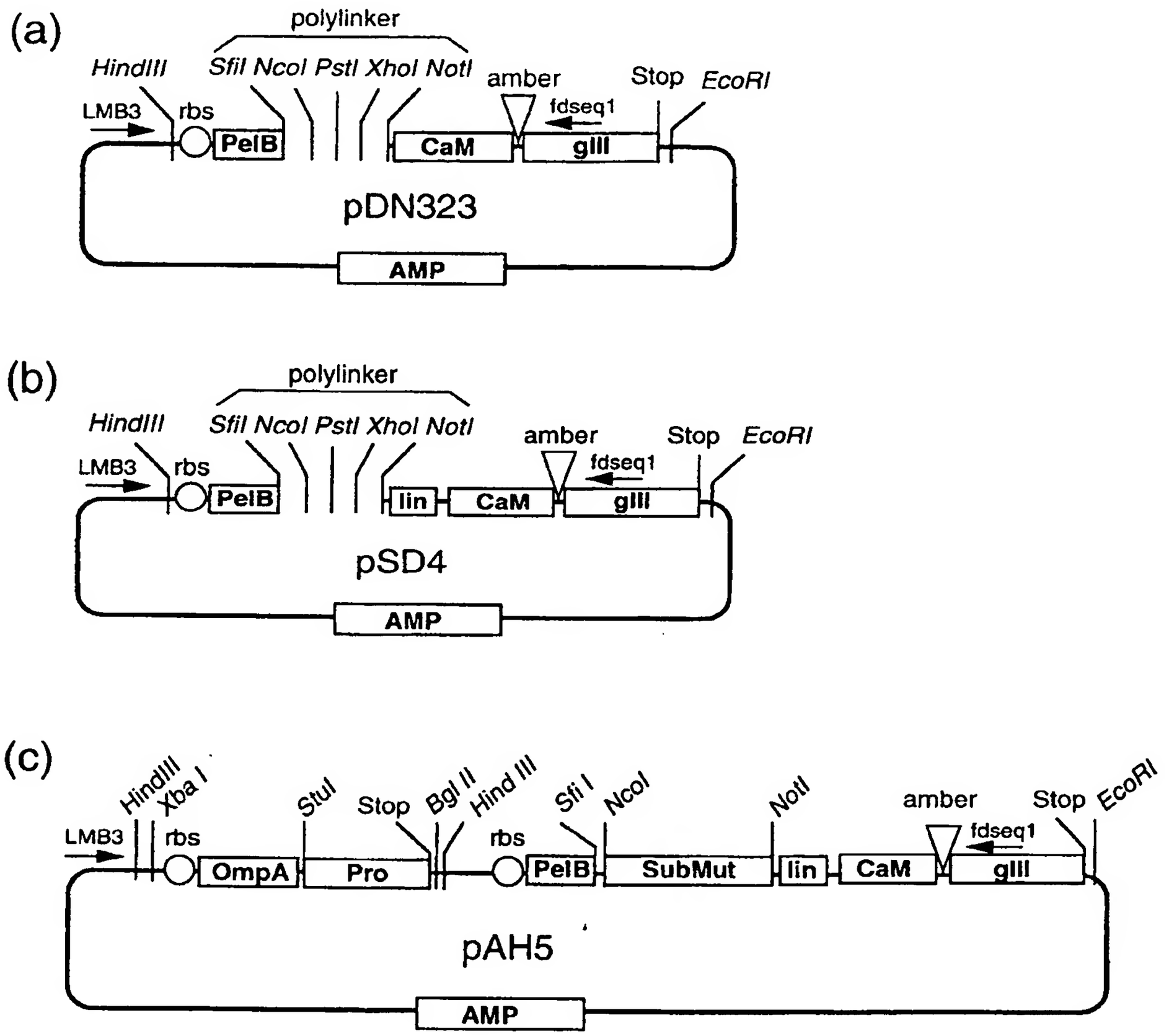


Figure 2. Phagemid vectors. Schematic representation of phagemids pDN323, pSD4 and pAH5, for the display of calmodulin-tagged enzymes on filamentous phage. Relevant single restriction sites are indicated. Rbs, ribosome binding site; PelB, leader peptide; OmpA, leader peptide; lin, polypeptide linker; CaM, calmodulin; gIII, gene III; AMP, ampicillin resistance gene. The approximate location of LMB3 and fdseq1 priming sites is indicated (see also Materials and Methods).

Calmodulin and specific peptide derivatives form a kinetically stable complex on filamentous phage

The selection methodology depicted in Figure 1 crucially depends on the formation of a stable complex between the calmodulin-binding peptide derivative and calmodulin. We tested the stability of this interaction in two ways.

In parallel reactions, we incubated phages displaying calmodulin with biotin-labelled high-affinity (biotin-CAAARWKKAFIAVSAANRFKKIS) and medium-affinity (biotin-CAAARAKKNFIAVSAANRFKKIS) calmodulin-binding peptides (Montigiani *et al.*, 1996). We then added a molar excess of peptide CAAARWKKAFIAVSAANRFKKIS and monitored the time-course of the competition reaction by ELISA, capturing phage particles on a streptavidin-coated plate. The results, illustrated in Figure 4(a), show that only the high-affinity binding peptide forms a complex stable for more than two hours ($k_{off} < 10^{-4}\ s^{-1}$).

The stability of the calmodulin-peptide (CAAARWKKAFIAVSAANRFKKIS) complex at pH values between 4 and 10 was also investigated by competition experiments with electrochemiluminescent detection (Neri *et al.*, 1996; Pini *et al.*, 1998). Over this pH range, the complex was stable ($k_{off} < 10^{-4}\ s^{-1}$; data not shown) suggesting that

Table 1. Peptides and proteins displayed on calmodulin-tagged filamentous phage

Protein displayed	Phagemid	Cloning[a]	Capturing reagent for ELISA
Linear FLAG peptide	pFV5	a	(1) CBP (2) Anti-peptide MAb
Anti-lysozyme scFv antibody fragment D1.3	pSM5	a	(1) CBP (2) Hen egg lysozyme
Glutathione S-transferase (GST)[a]	pSD8	a,b[a]	(1) CBP (2) Anti-GST MAb
Subtilisin mutant ("submut")	pAH5	c	(1) CBP (2) Chymotrypsin inhibitor 2
Catalytic tyrosine kinase domain of human insulin receptor (IRK)	pAH1	a	(1) CBP
Catalytic tyrosine kinase domain of human *lck*	pSM6	a	(1) CBP (2) Anti-*lck* PAb

Reagents for phage ELISA: CBP, biotinylated calmodulin binding peptide biotin-CAAARWKKAFIAVSAANRFKKIS (Montigiani *et al.*, 1996) immobilised on streptavidin-coated plate; MAb, monoclonal antibody; Pab, polyclonal antiserum. Phage ELISA assays were performed according to standard methodologies (Nissim *et al.*, 1994), using a commercially available anti-M13-horseradish peroxidase conjugated polyclonal antibody as detecting reagent for the phage particles.

[a] See also Figure 7 and the Discussion.

[b] The phagemid was obtained by inserting in (a) pDN323, (b) pSD4 or (c) pAH5 the gene coding for the protein to be displayed on phage (see Figure 2 for schematic representation of the phagemid vectors).

our methodology should be applicable to a variety of different experimental conditions.

In many enzyme-catalysed reactions it is important to use reagents at relatively high concentrations (comparable to the Michaelis-Menten constant), which may have to be removed from the phage solution before the capture step (Figure 1; see below), as they might interfere with the selection efficiency. A solution to this problem would be to separate the phage particles from small organic molecules by selective precipitation in the presence of polyethylene glycol (PEG), a procedure that requires sufficient stability of the calmodulin-peptide complex during the precipitation step.

We performed such precipitation assays, and monitored the efficiency of the methodology by ELISA and by phage titre determination. The results, reported in Figure 4(b), indicate that the calmodulin-biotin-CAAARWKKAFIAVSAANRFKKIS complex on phage is stable even after PEG precipitation.

Calmodulin-tagged enzymes on phage can efficiently be selected and amplified using reaction products covalently bound to a calmodulin-binding peptide

Selecting for catalysis according to the scheme of Figure 1 requires affinity reagents which are specific for the reaction product. We used products of two model enzymatic reactions to demonstrate that phage particles carrying the reaction product anchored on their surface *via* the calmodulin tag can be rescued and amplified using suitable affinity reagents. We chose as model enzymes: (i) the endopeptidase "submut" (a subtilisin mutant; Carter & Wells, 1987; Table 1), which cleaves peptides containing the AAHY sequence (Figure 5a), and (ii) glutathione-S-transferase (GST) from *Schistosoma japonicum*, that catalyses the formation of a covalent carbon-sulphur bond between glutathione and reactive aromatic halide groups (Mannervik & Danielson, 1988).

Biotin-CAAARWKKAFIAVSAANRFKKIS may represent the product of a bimolecular covalent

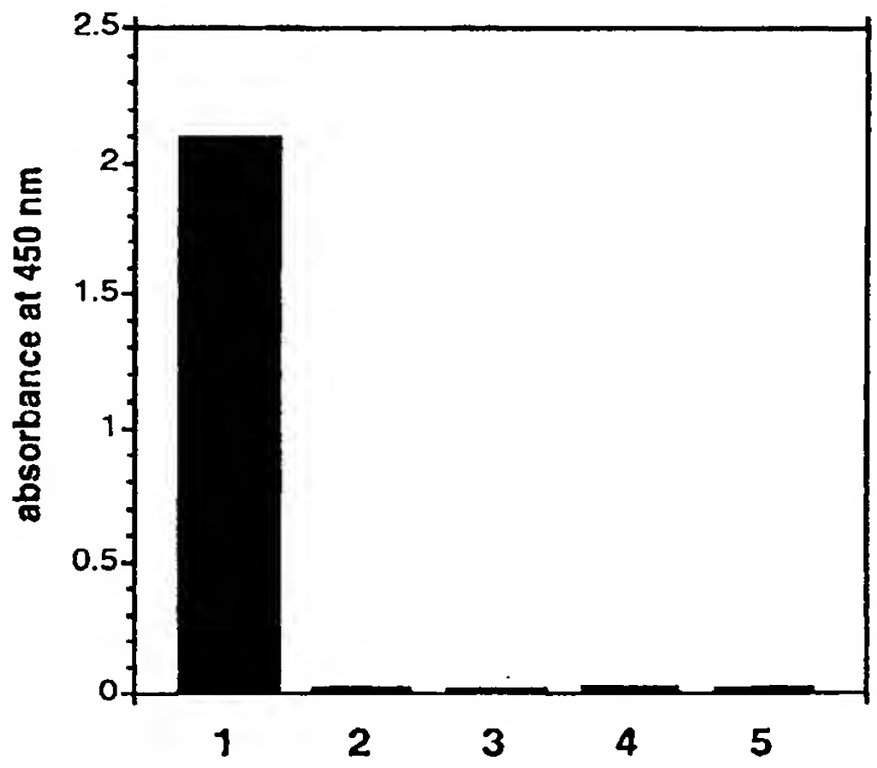


Figure 3. Functional evaluation of calmodulin-tagged phage. ELISA results obtained with glutathione-*S*-transferase displayed on calmodulin-tagged phage (clone pSD8; Table 1) and biotin-labelled CAAARWKKAFIAVSAANRFKKIS peptide. GST-CaM-phage showed binding in the presence (1), but not in the absence (2) of the biotinylated peptide. The calcium chelator EDTA also abolished binding (3). A non-specific phage (4; GST-phage from phagemid pFV7, Materials and Methods) and milk (5) were also negative. Similar results were reproducibly obtained with other calmodulin-tagged phages (Table 1).

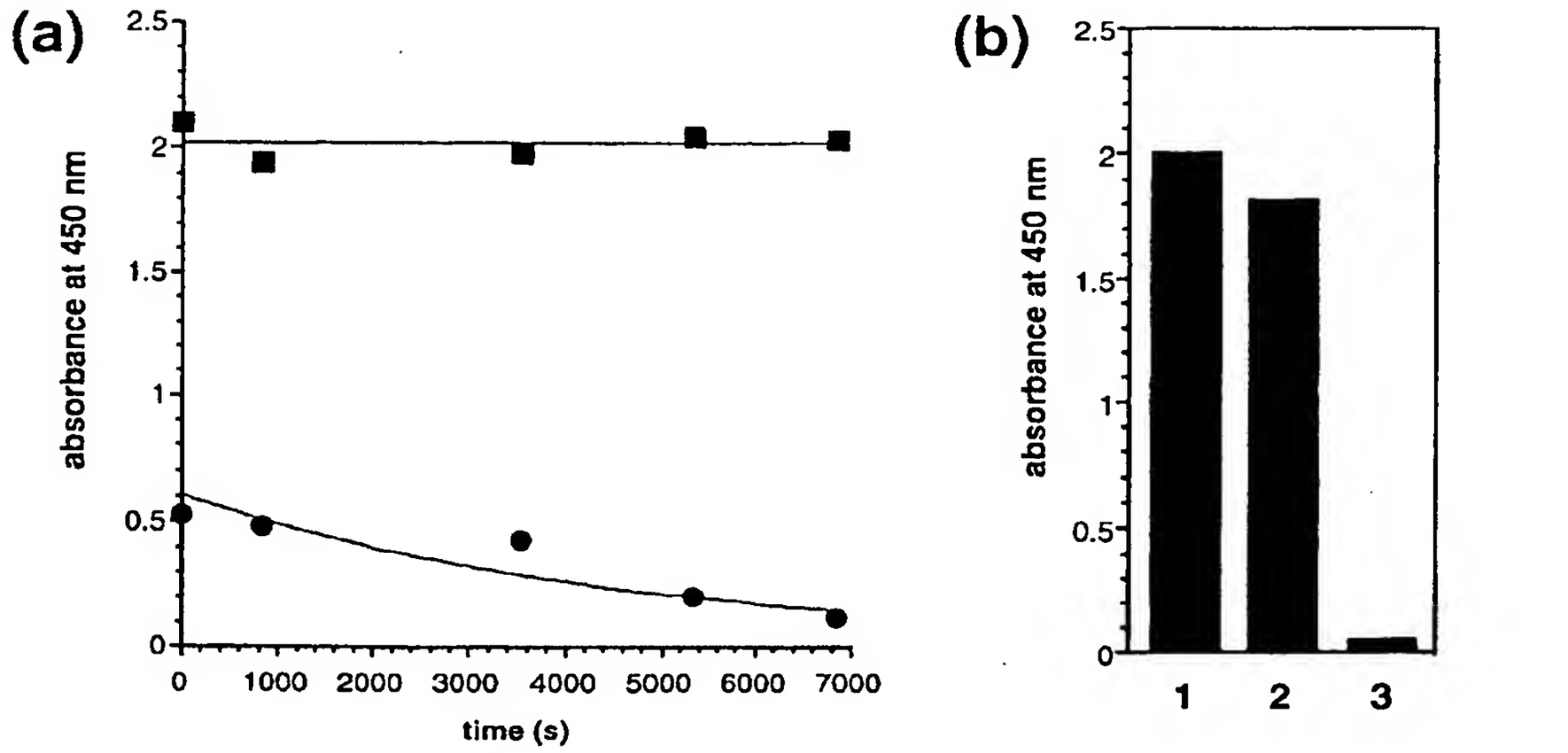


Figure 4. Stability of calmodulin-tagged phage/peptide complexes. (a) Competition of (calmodulin-phage)/(biotin-peptide) complex with a 1000-fold molar excess of non-biotinylated calmodulin-binding peptide, detected by ELISA. Squares; CAAARWKKAFIAVSAANRFKKIS (peptide with high affinity for calmodulin); circles, CAAARAKKNFIAVSAANRFKKIS (moderate affinity for calmodulin; Montigiani *et al.*, 1996). (b) Stability of (calmodulin-phage)/(biotin-CAAARWKKAFIAVSAANRFKKIS) complex after precipitation in PEG, detected by ELISA on streptavidin-coated plate. The ELISA signals before (1) and after (2) PEG precipitation are comparable. This result is consistent with the negligible loss of phage titre after PEG precipitation. In sample 3, 2% milk in TBSC was used instead of phage. Calmodulin-phage was produced from phagemid pDN323 (Figure 2).

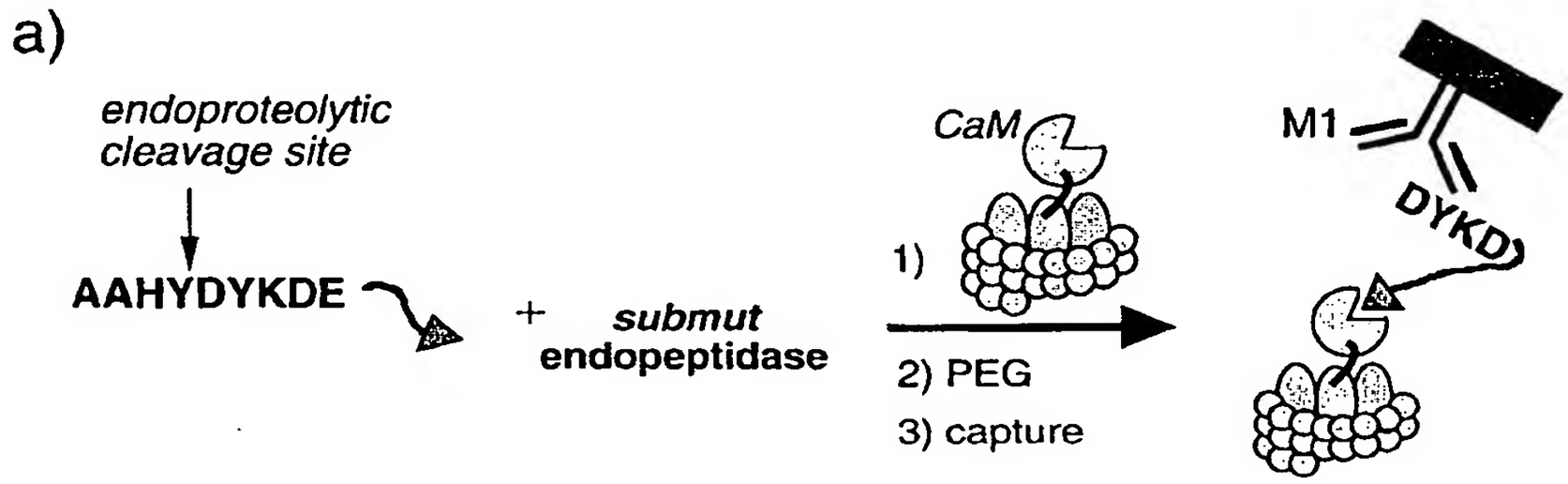


Figure 5. Selection schemes with reaction products anchored on phage *via* the calmodulin tag. In these schemes, reaction products were generated by addition of purified enzymes to the reaction mixture, followed- by capture on calmodulin phage. (a) The endopeptidase submut cleaves the peptide between Y and D. The resulting peptide is captured with the monoclonal antibody M1, specific for peptides carrying the sequence DYKDE at their N terminus. (b) GST catalyses the nucleophilic attack of the thiol group of glutathione (fused at the N-terminal extremity of a calmodulin binding peptide; see the text) on 1-chloro-2,4-dinitrobenzene. The resulting reaction product is captured by a specific scFv antibody fragment.

Table 2. Enrichment of enzyme-calmodulin-fd phage over enzyme-fd phage fusions by capture with a biotin-labelled calmodulin-binding peptide and streptavidin-coated magnetic bead

Input				Output		
Titre A E-CaM-fd[a] (t.u./ml)	Titre B E-fd[a] (t.u./ml)	Ratio A/B	Rounds of Selection (enzyme E)	Titre (t.u./ml)	Ratio[b] A/B	Enrichment
5×10^7	5×10^{10}	$1:10^3$	1 (GST)	7×10^5	40:0	$>4 \times 10^4$
3×10^8	3×10^{11}	$1:10^3$	1 (IRK)	5×10^5	26:1	2.6×10^4
1×10^6	1×10^{12}	$1:10^6$	2 (GST)	5×10^7	20:0	$>2 \times 10^7$
5×10^4	5×10^{10}	$1:10^6$	2 (GST)	2×10^7	20:0	$>2 \times 10^7$
6×10^3	6×10^9	$1:10^6$	2 (IRK)	1×10^5	15:4	3.8×10^6
6×10^5	6×10^{11}	$1:10^6$	2 (IRK)	5×10^5	11:8	1.4×10^6

Biopanning experiments were performed as described Materials and Methods. "Input" phages are those used for the selection; "output" phages are those recovered after the biopanning procedure (but before *E. coli* amplification).

[a] Phages prepared from pSD3 and pFV7, respectively (Materials and Methods). E is enzyme; GST is glutathione-*S*-transferase; IRK is the tyrosine kinase domain of the insulin receptor; CaM is calmodulin.

[b] The sum of A+B is equal to the total amount of colonies screened by PCR.

bond-forming reaction, in which one of the two reagents is coupled to the peptide and the other reagent is biotinylated (see below).

As summarised in Table 2, phage displaying the GST-calmodulin fusion was mixed with a 1000-fold molar excess of phage displaying only GST, and incubated with biotin-CAAARWKKAFIAV-SAANRFKKIS. The resulting mixture was then selected using streptavidin-coated magnetic beads and amplified by bacterial infection. A similar selection experiment was performed with phages displaying a tyrosine kinase (Table 2). PCR screening of infected bacterial colonies revealed an enrichment factor of at least 20000:1 in a single round of panning. Selection of calmodulin-tagged phage at greater dilutions ($1:10^6$; Table 2) led to an enrichment factor greater than 10^6:1 after two rounds of panning. This good selectivity is the result of the high-affinity capture reagent (streptavidin) and of the mild and selective elution protocol used (calcium chelation by EDTA).

Peptide DYKDEGGGAAARWKKAFIAVSAAN-RFKKIS represents the cleavage product of a longer peptide **R**-DYKDEGGGAAARWKKAFIAV-SAANRFKKIS, in which the peptidic sequence **R** is removed by an endopeptidase.

The monoclonal antibody M1 recognises peptides carrying the DYKDE sequence at their N-terminal extremity, but not peptides with the DYKDE sequence at internal positions (Hopp *et al.*, 1988).

We incubated calmodulin-tagged phage displaying the endopeptidase submut in the presence or in the absence of 100 nM DYKDEGGGAAARWK-KAFIAVSAANRFKKIS. We then removed the excess peptide by precipitation with PEG, and captured the phage particles with biotin-labelled M1 antibody immobilised on streptavidin-coated magnetic beads. Titres of phage recovered were reproducibly >100-fold higher when the phage had been incubated with the peptide.

The results described in above were obtained not only with purified reaction products, but also by *in situ* generation of products by addition of purified enzyme to the reaction mixture, as depicted in Figure 5 (see Materials and Methods). In negative control experiments, no enzyme was added.

For GST, several capture strategies could be envisaged (see below for a more detailed description). A possible strategy features the use of a monoclonal antibody as affinity reagent (Figure 5(b)), which has been isolated from a synthetic antibody phage display library, and which is specific for *S*-(2,4-dinitrophenyl)glutathione (Figure 6). Enrichment factors relative to

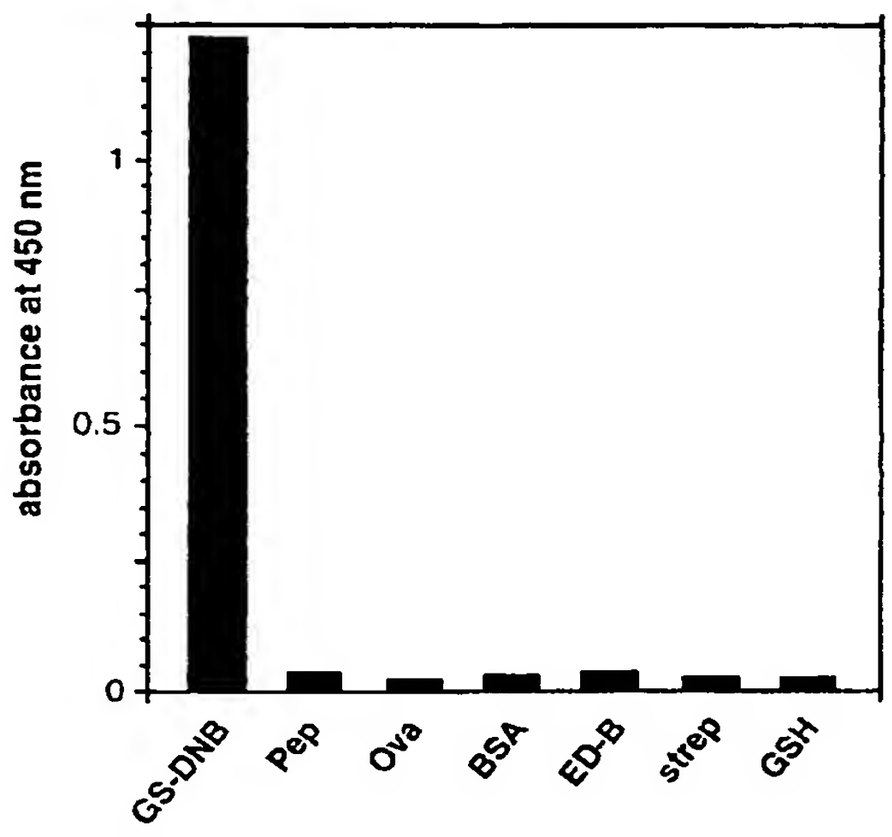


Figure 6. Reactivity of a recombinant antibody fragment: specific for *S*-(2,4-dinitrophenyl)-glutathione. ELISA reactivity of the recombinant antibody G3, isolated from a synthetic antibody phage library, against *S*-(2,4-dinitrophenyl)-glutathione and irrelevant antigens. Note that the antibody reacts with biotinylated *S*-(2,4-dinitrophenyl)-glutathione (Cl-DNP), but not with the phenylalanine-containing peptide CAAARWKKAFIAV-SAANRFKKIS (Pep). Other antigens: Ova, ovalbumin; BSA, bovine serum albumin; ED-B, ED-B domain of fibronectin, strep, streptavidin; GSH, biotinylated glutathione captured on streptavidin-coated plate.

the negative controls were 300 for submut, and 2200 for GST.

Model selection experiments with the endopeptidase submut

Display of submut on calmodulin-tagged phage

One of our long-term goals is the development of novel sequence-specific endopeptidases. In order to study the selection strategy of Figure 1 in this context, we performed selection experiments using a specific endopeptidase, submut, as the model enzyme. Submut is a mutant of subtilisin, in which the catalytically relevant histidine-64 residue has been replaced by alanine (Carter & Wells, 1987), and which has been engineered to facilitate display on phage (see below). An analogue of submut, "genenase I", has been used for a variety of biochemical applications (Ward *et al.*, 1996) and is commercially available.

The zymogen of subtilisin and of subtilisin mutants contains an inhibitory Pro domain located N-terminally, which is cleaved to yield the mature protease. The Pro peptide is reported to be essential for protease folding. However, unfolded mutant subtilisin can be refolded by the addition of purified Pro peptide (Strausberg *et al.*, 1993). Deletion of a "calcium loop" in subtilisin further increases the rate of subtilisin folding (Gallagher *et al.*, 1993). The mutation of alanine 73 to leucine was also reported to increase protein stability (Strausberg *et al.*, 1995).

In order to satisfy the folding requirements of subtilisin, we constructed the bicistronic phagemid vector pAH5 (Figure 2), which expresses separately the Pro peptide and the mature subtilisin mutant fused to calmodulin-pIII (Table 1).

Catalysis-mediated enrichment of submut-CaM-phage particles

In order to evaluate whether submut-CaM-phage can be enriched for catalysis, we performed selections incubating the 10^{10} phage particles with three different peptides containing the DYKDE sequence (boldface): **1**, **DYKDE**GGGAAARWK-KAFIAVSAANRFKKIS (positive control); **2**, GAA-HY**DYKDE**GGGAAARWKKAFIAVSAANRFKKIS (substrate of submut); and **3**, GDDDDK**DYK-DE**GGGAAARWKKAFIAVSAANRFKKIS (negative control).

After precipitation with PEG, 3×10^7 **(1)**, 1×10^6 **(2)** and 9×10^3 **(3)** phages were rescued, respectively, using the M1 antibody as capture reagent. Enrichments of peptide **2** over peptide **3** was not observed when GST-CaM-phage was used.

On the basis of the k_{cat} value of submut (10^{-2} s^{-1}; Carter *et al.*, 1989), we can calculate that the reaction time used for the selection (15 minutes) was sufficiently long for mediating one intramolecular conversion on phage. The results obtained indicate that submut-CaM-phage can be enriched when a specific peptide is used. However, the fact that more phage is rescued when the positive control peptide **(1)** is used, suggests that either the enzyme is not completely active on phage, or that submut is less abundant than calmodulin on phage (as would be the case if proteolysis between submut and calmodulin takes place).

To further demonstrate that submut-CaM-phage can be selected by virtue of its catalytic activity, we incubated submut-CaM-phage and GST-CaM-phage in 0.9:1 ratio in the presence of peptide **(2)**, and we captured the phage as described above. After selective elution, phage was used to infect *Escherichia coli* bacteria. After this single round of selection, PCR screening of individual colonies showed that the ratio of submut:GST phages was 49:1 (enrichment factor = 54).

Incubation of submut-CaM-phage and GST-CaM-phage with the negative control calmodulin-binding peptide **(3)**, which is not a substrate of submut, resulted in a slight enrichment of GST phage over submut phage (enrichment factor = 1.1).

We anticipate that increased enrichment factors could be obtained with endopeptidases that are better expressed on phage, and which have fewer folding requirements, for example the cyclophilin mutant "cyproase" (Quemeneur *et al.*, 1998; unpublished observation).

Model selection experiments with glutathione-*S*-transferase from *S. japonicum*

A number of other parameters can influence the efficiency of the selection of phage enzymes according to the scheme of Figure 1. To study the effect of these parameters, we have chosen glutathione-*S*-transferase from *S. japonicum*, as a model enzyme that catalyses a bimolecular reaction.

Display of GST on calmodulin-tagged phage

GST is enzymatically active only as a dimer. One way to promote the assembly of dimeric GST onto the minor coat protein pIII of filamentous phage is to promote the heterodimerisation between GST-CaM-pIII fusion and GST in the periplasmic space. In order to have GST and GST-fusion encoded by the same vector, we have exploited the property that the amber codon is read either as a stop or as a glutamic acid by suppressor strains such as TG1 *E. coli* bacteria (Hoogenboom *et al.*, 1991).

We have prepared and characterised four constructs that encode different formats of GST-calmodulin fusions on phage (Figure 7). The phage titres obtained and the ELISA results indicated that GST-calmodulin-pIII fusions can be produced with different sequential orders of the GST-calmodulin genes (Figure 7).

Western blot analysis with phage preparations obtained from phagemid pSD3 and pSD8 (Figure 7) had indicated that the GST-CaM-pIII fusion protein was poorly incorporated into the phage compared

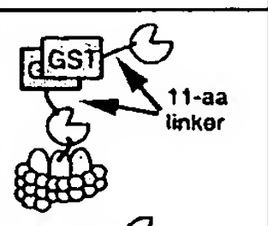
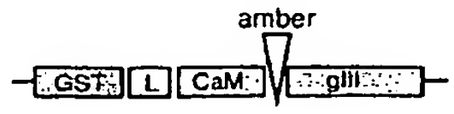
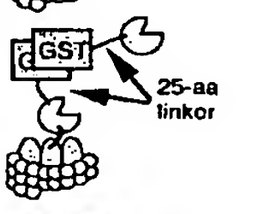
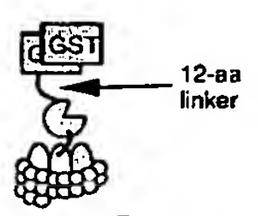
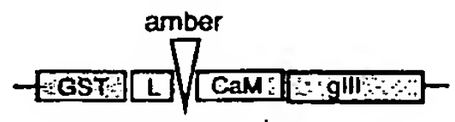
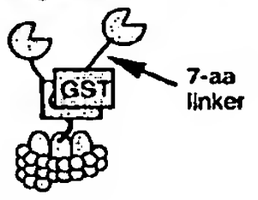
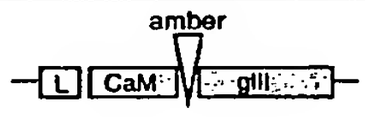
vector	schematic structure	phagemid insert	ELISA CaM	ELISA GST	titre (VCS-M13) [tu/ml]	titre (KM13) [tu/ml] - trypsin	titre (KM13) [tu/ml] + trypsin
pSD3			++	++	6×10^{12}	4×10^{12}	6×10^{9}
pSD8			++	++	1×10^{13}	3×10^{12}	2×10^{9}
pFV68			++	++	4×10^{12}	n.d.	n.d.
pFV67			++	++	5×10^{12}	1×10^{13}	4×10^{9}
pSD4			++	-	8×10^{12}	7×10^{12}	1×10^{11}

Figure 7. Formats of GST-calmodulin fusion proteins displayed on phage. Phagemid vectors that promote the display of different formats of GST-calmodulin fusions on phage. pSD4 is also presented, as reference phagemid (Figure 2). Phage ELISA was performed as in Table 1. The corresponding phage particles were produced using two different helper phages: VCS-M13 and KM13. Phages produced with VCS-M13 had identical titre, even if they were treated with trypsin before bacterial infection. The loss in titre of phages produced with the "cleavable" KM13 helper phage after incubation with trypsin corresponds to the phages that did not display the GST-CaM-pIII fusion on their surface (Kristensen & Winter, 1998).

to the pIII protein (data not shown). This appeared to reflect better incorporation of pIII from the helper phage, as shown by the use of a helper phage (KM13) in which the pIII protein of the helper phage (but not that of fusion protein) can be cleaved with trypsin so as to render it incapable of mediating infection (Kristensen & Winter, 1998). Thus, after proteolysis only those phages that had incorporated the fusion protein should be infective.

From the loss in titre after proteolysis we estimated that approximately only one phage particle in a thousand had incorporated the GST-CaM-pIII fusion protein (Figure 7). Phages displaying only calmodulin showed a better incorporation of pIII fusion protein (1:70; pSD4 in Figure 7).

The lower expression of GST-CaM-pIII on phage, compared to CaM-pIII, was also evidentiated by ELISA experiments performed with serial dilutions of phage particles, using biotinylated calmodulin-binding peptide as capture reagent (data not shown)

Selection strategy and preparation of reagents

The model selection experiments described above suggest that a suitable reaction scheme for the enzymatic selection of GST-CaM-phage could be the one depicted schematically in Figure 8.

Inspection of the three-dimensional structure of GST in the complex with the reaction product between glutathione and 1-chloro-2,4-dinitrobenzene revealed that the C terminus of glutathione is solvent exposed. This suggests that it should be possible to produce peptides that contain glutathione at the N terminus and which remain good substrates of GST. Using solid phase synthesis methodologies, we prepared the peptide γGlu-Cys-Gly-GGGAAARWKKAFIAV-SAANRFKKIS (glutathione-PEP), which contains glutathione (indicated with three-letter code amino acid symbols), fused to the calmodulin-binding peptide. Spectrophotometric analysis of the reaction between glutathione-PEP and 1-chloro-2,4-dinitrobenzene catalysed by GST (Widersten & Mannervik, 1995; Habig *et al.*, 1974) confirmed that glutathione-PEP and glutathione are substrates of comparable quality for the reaction.

We then investigated whether 1-chloro-3,5-dinitrobenzene derivatives substituted in position 1

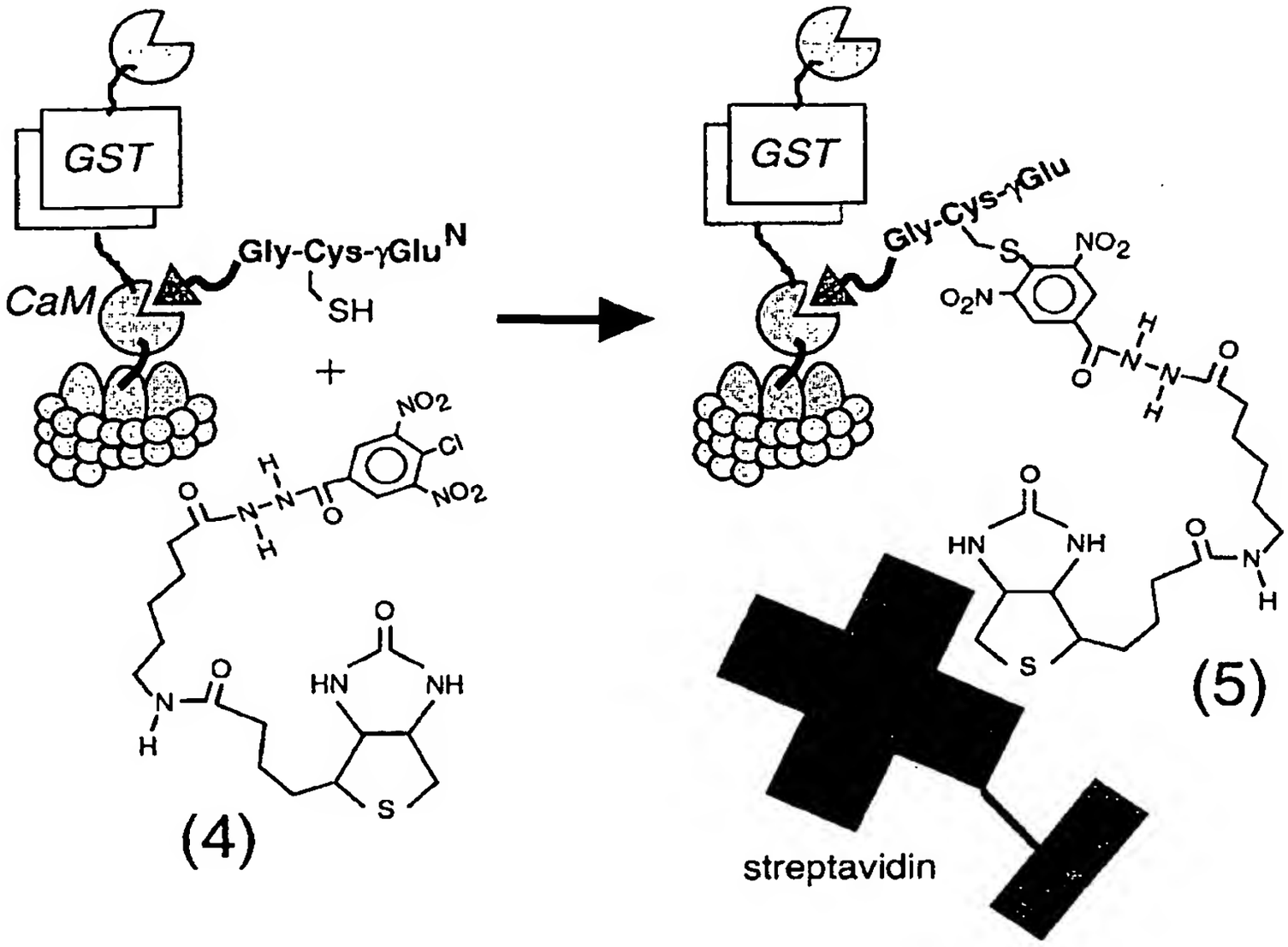


Figure 8. Selection strategy for the catalytic activity of GST displayed on calmodulin-tagged phage. The selection scheme features the display of dimeric GST as calmodulin fusion on phage. Glutathione, fused N-terminally to a calmodulin-binding peptide which anchors it on phage, reacts with the biotinylated derivative of 4-chloro-3,5-dinitrobenzoic acid (**4**). Phage particles carrying the biotinylated calmodulin-bound reaction product (**5**) are isolated by capture on streptavidin-coated magnetic beads, and eluted selectively with the calcium chelator EDTA.

were good substrates for the GST-catalysed reaction with glutathione. The reaction parameters measured spectrophotometrically for 4-chloro-3,5-dinitrobenzoic acid ($k_{cat} = 0.74\ s^{-1}$; $k_{uncat} = 3 \times 10^{-2}\ s^{-1}\ M^{-1}$; K_M (4-chloro-3,5-dinitrobenzoic acid) = 0.9 mM; K_M (glutathione) = 0.3 mM; T = 25 °C and pH = 7.2) were comparable to those reported for the GST-catalysed reaction between glutathione and 1-chloro-2,4-dinitrobenzene (Mannervik & Danielson, 1988; Hansson *et al.*, 1997).

On the basis of these reaction parameters, upon incubation of phage carrying the glutathione-PEP peptide bound to calmodulin with 1 mM 4-chloro-3,5-dinitrobenzoic acid derivative, one can calculate that: (i) the GST-catalysed reaction of Figure 8 should proceed to completion in less than two seconds; (II) in four seconds, only 1:10,000 phages displaying glutathione on their surface react in an uncatalysed fashion with 1 mM 4-chloro-3,5-dinitrobenzoic acid derivatives.

We therefore synthesised the biotinylated dinitrophenyl derivative (substrate 4, Figure 8), and confirmed spectrophotometrically, by ELISA and by HPLC analysis, that it reacts in a GST catalysed manner with glutathione-PEP (data not shown).

The thiol group of a glutathione-peptide derivative is reactive when anchored on phage

The thiol group of glutathione-PEP peptide, anchored on phage *via* the calmodulin tag (Figure 8) could, in principle, be prone to oxidation, thereby losing its reactivity. To demonstrate that the thiol group remains reactive when glutathione-PEP is bound to the phage, we treated the GST-CaM phage with 1 mM *N*-[6-(biotinamido)hexyl-3'-(2'-pyridyldithio)propionamide (biotin-HPDP), a biotinylating reagent specific for cysteine residues. Phage was then captured with streptavidin-coated magnetic beads, and eluted with dithiothreitol (DTT) or with EDTA. The results of this experiment are summarised in Table 3.

Comparable titres of phage were recovered by EDTA elution with a biotinylated peptide, or when glutathione-PEP was biotinylated on phage by reaction with biotin-HPDP. In the absence of glutathione-PEP, the titre recovered was significantly lower.

The high background of phage recovered by DTT elution confirms that several reactive cysteine residues (other than glutathione) are directly present on phage, or on proteins tightly associated with phage (Pedersen *et al.*, 1998; Jestin *et al.*, 1999;

Table 3. Recovery of GST-calmodulin-phage-peptide complex after treatment with biotin-HPDP

Experiment	Pep-GSH	Pep-biotin	Biotin-HPDP	Titre recovered[a] DTT	EDTA
a	+	−	+	5×10^7	1.1×10^8
b	−	−	+	3×10^7	7×10^6
c	−	+	−	n.d.	2.5×10^8
d	−	+	+	n.d.	1.3×10^8

Biopanning experiments were performed as described in Materials and Methods; 10^{11} phages GST-calmodulin-phage produced from phagemid pSD8 were used as input for the selection experiments, after incubation either with γGlu-Cys-Gly-GGGAAARWK-KAFIAVSAANRFKKIS (pep-GSH) or biotin-CAAARWKKAFIAVSAANRFKKIS (pep-biotin; Montigiani *et al.*, 1996).

[a] After reaction with biotin-HPDP, phages were precipitated with PEG, captured with streptavidin-coated magnetic beads and eluted either with 20 mM DTT or with 20 mM EDTA in TBS, and titred by bacterial infection.

S.D. & D.N., unpublished results). The thiol groups of cysteine residues could, in principle, be used as target for chemical modification of phage with reaction substrates, but other cysteine residues of phage would also react. In this respect, targeting of calmodulin-tagged phages with specific peptides (Figure 1) appears to be a more selective and possibly a more attractive avenue for the anchoring of reaction substrates/products on phage in proximity of the enzymatic active site.

Model selection experiments using a glutathione derivative anchored on phage and a biotinylated reactive aromatic halide

We used phage displaying GST as CaM-pIII fusion, prepared from phagemid pSD8 (Figure 7), as a model to test the selection scheme depicted in Figure 8.

A total of 10^{10} phage particles were incubated with glutathione-PEP peptide alone, with the biotinylated aromatic chloride (4) alone, or with a mixture of the two reagents. As a positive control, biotin-CAAARWKKAFIAVSAANRFKKIS was used, as described above. The phage titres rescued in the selection procedure are reported in Figure 9. The titre obtained when both substrates were used in the reaction mixture (a), was at least 60-fold higher than the titre obtained when only one reaction substrate was used (b-d).

This suggests that the thioether bond between glutathione and biotinylated aromatic chloride was indeed formed on GST-calmodulin displayed on phage. Approximately 15% of this reaction

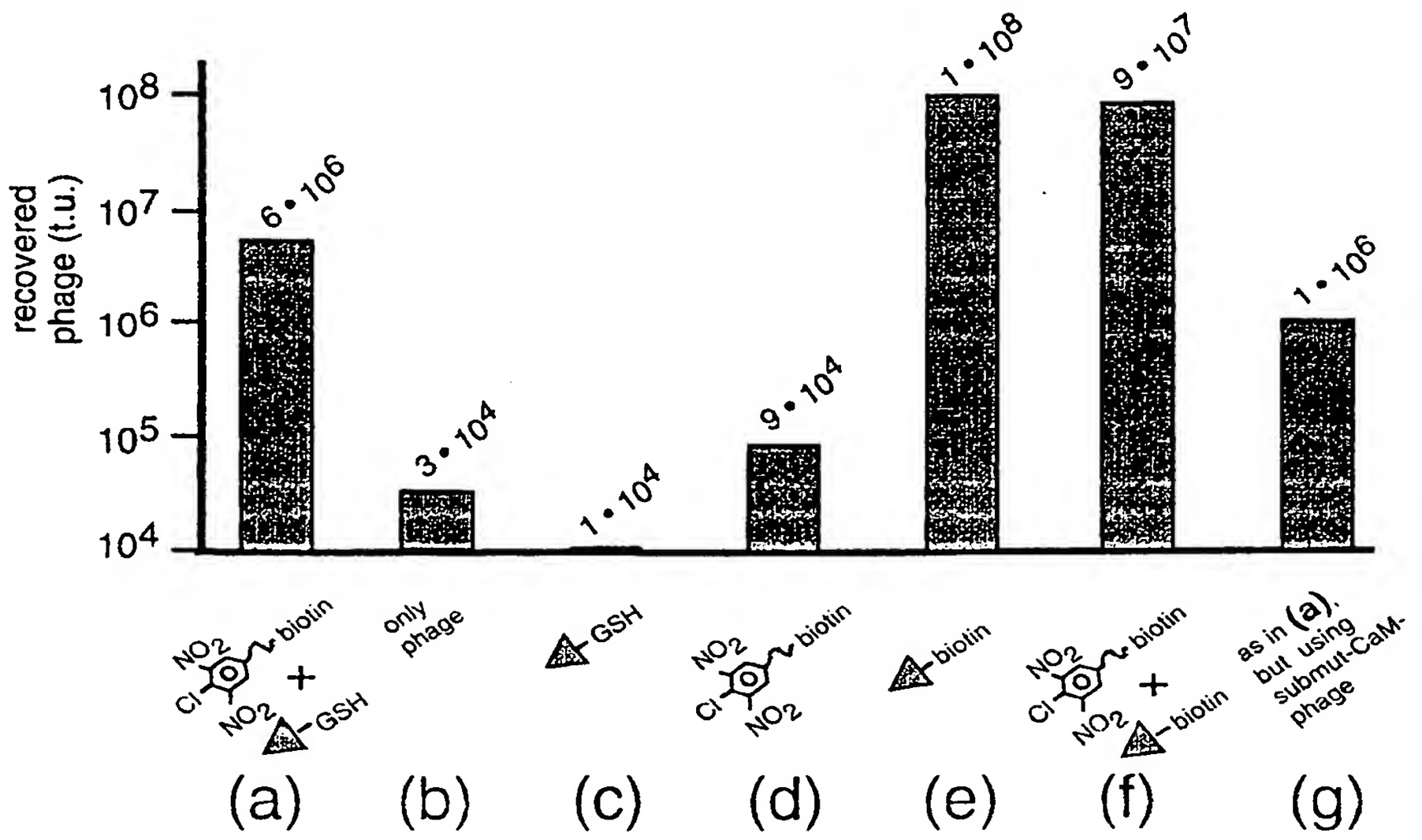


Figure 9. Model selections with GST-CaM phage. Recovery of GST-CaM-phage (from phagemid pSD8), in reactions that feature different combinations of the substrates, according to the selection scheme in Figure 8.

proceeded in an uncatalysed fashion, as suggested by the titre rescued when using phage displaying an irrelevant enzyme (submut) as CaM-pIII fusion (g). Furthermore, the significantly larger amount of phage recovered with the positive control biotinylated peptide (e,f) suggests that *S. japonicum* GST on phage is either partially inactive, or partially lost by proteolysis between GST and calmodulin.

Discussion

The design and synthesis of novel enzymes has been defined as "the ultimate achievement in enzymology" (Fersht, 1984). This goal has become more approachable with the development of protein engineering (Winter *et al.*, 1982; Wilkinson *et al.*, 1984). Born by the combination of the powerful advances in structural biology and genetic engineering, protein engineering has allowed the production of site-directed mutant enzymes, and the dissection of the role of individual amino acids in catalysis. In some cases, protein engineering techniques have resulted in the isolation of mutant enzymes with improved stability and/or catalytic performance (Wells *et al.*, 1987; Wilkinson *et al.*, 1984), or even with changes in enzyme substrate specificity (see, for example, Rutter *et al.*, 1987; Carter & Wells, 1987; Wu & Hilvert, 1989; Zhu *et al.*, 1995; Widersten & Mannervik, 1995; El Hawrani *et al.*, 1996; Feil *et al.*, 1997; Quemeneur *et al.*, 1998; Liu *et al.*, 1998). Three-dimensional structures of enzymes facilitate the selection of residues to be mutated, on the basis of their geometric and chemical relation to the enzyme's active site. Yet it is difficult to make full use of this powerful information, because of the time and work needed to clone, express and characterise mutant enzymes. Combinatorial mutagenesis (Reidhaar-Olson & Sauer, 1988) may facilitate the task, but unless efficient screening methodologies are available (for example, direct screening for enzymatic activity of bacterial colonies on agar plates; El Hawrani *et al.*, 1996; Tang *et al.*, 1991), the production and characterisation of individual clones may limit the efficiency of the procedure.

Protein repertoires displayed on filamentous phage promise to be useful tools for the isolation of novel enzymatic activities. This goal is potentially achievable by anchoring reaction substrates on the surface of enzyme-displaying filamentous phage, in a way that they can reach the enzyme's active site. Physical isolation of those phage enzymes which convert the substrate into the desired product could then be possible with affinity reagents specific for the reaction, therefore allowing a direct selection for catalysis.

As a first step towards the isolation of enzymatic activities from large protein repertoires, we have developed a methodology for the conditional anchoring of reaction substrates and products on phage, based on the calmodulin-ligand complex system (Neri *et al.*, 1997; Montigiani *et al.*, 1996). Using glutathione-*S*-transferase and an endopeptidase as model systems, we have shown that enzymes displayed on calmodulin-tagged phage can be selected by virtue of their catalytic activity with enrichment factors greater than 50 per single round of selection.

We expect that the efficiency of selections for catalysis could be improved if more enzyme can be displayed on phage (on average, GST or submut are displayed on 1:100-1:1000 phage particles, using the phagemids of Figures 2 and 7). The choice of enzymes with improved folding properties, or the use of phage (rather than phagemid) vectors is likely to result in enhanced enrichment factors. However, low levels of display of antibody fragments on phage using phagemid vectors has not precluded the development of antibody phage technology as a powerful alternative to hybridoma technology. Indeed, with three rounds of selection/amplification, binding specificities against virtually any antigen can be rescued from large single-pot antibody libraries (Winter *et al.*, 1994; Marks *et al.*, 1991; Nissim *et al.*, 1994; Griffiths *et al.*, 1994; Vaughan *et al.*, 1996; Pini *et al.*, 1998).

A possible disadvantage of the reaction scheme of Figure 1 is that a single conversion of substrate into product occurs for each phage. It may, therefore, be difficult to discriminate between two enzymes with different kinetic properties. The stringency of the selection for catalysis (i.e. the discrimination between catalysts of different quality) could however, be influenced by the concentration of reagents used, the choice of the solvent, the linker length, the use of competitors and by the reaction time and temperature.

It is also worth mentioning that a reaction *in trans*, with one phage enzyme catalysing the conversion of the substrate anchored on a different phage particle, would be extremely unlikely. In fact, selections are typically performed at phage concentrations below 10^{12} t.u./ml, which corresponds to an upper-limit concentration for both phage-enzyme and substrate in the nanomolar range. In these conditions, most catalysed reactions do not proceed.

The reaction scheme of Figure 1 crucially relies on the availability of affinity reagents which efficiently distinguish between reagent and product. This degree of specificity could be provided by antibodies (either produced by hybridoma or phage technology; Winter *et al.*, 1994), by single-stranded nucleic acids (nexamers; Gold *et al.*, 1995), or by naturally occurring binders (streptavidin for biotinylated reaction products, lectins for oligosaccharides, SH2 domains for phosphotyrosine containing peptides, DNA-binding proteins for DNA, etc.).

For some reactions it may be necessary to incubate the enzyme phage library with reagents at concentrations comparable to the Michaelis con-

stant K_M of the enzyme. For example, in selections with GST-calmodulin-phage, we have used a biotinylated reaction substrate (4 in Figure 8) at concentrations in the millimolar range. Capture with streptavidin-coated beads of phages displaying the biotinylated reaction product would be inefficient, unless the excess of unreacted biotinylated substrate could be removed. The availability of rapid purification protocols for calmodulin-tagged phages, such as the PEG precipitation described in Figure 4(b), has proven to be useful for removing reagents in excess and for stopping enzymatic reactions.

Recently, Janda *et al.* (1997) described the direct selection of antibodies with glycosidic activity from a phage display library, by covalent phage trapping on a reactive support. The efficiency of this selection scheme can hardly be evaluated at the moment, as the authors used a small library (approximately 9000 clones), four rounds of selection, and did not comment on the percentage of enzymatically active phage particles before and after selection. The reaction scheme of Figure 1 is possibly more general, as it is compatible with a large variety of different enzyme reactions, and does not require knowledge of the reaction mechanism and particular chemical groups for phage trapping.

It is likely that both the selection scheme of Figure 1 and that of Pedersen *et al.* (1998) will be useful for selecting novel enzymes from large repertoires on phage. Calmodulin-tagged phages may have the advantage that elution can be performed in a mild and selective fashion by the addition of calcium chelators (Figure 1). Furthermore, the intramolecular reaction on phage may be facilitated by the use of a suitably long linker either between substrate and calmodulin-binding peptide, or between enzyme and calmodulin (Figure 1). An additional benefit may be that the calmodulin-ligand system allows one molecule of substrate per molecule of enzyme displayed on phage.

Materials and Methods

Peptides, antibodies and chemical reagents

Peptides and their biotinylated derivatives (modified at the thiol group of the N-terminal cysteine residue) were synthesised and HPLC-purified as described (Montigiani *et al.*, 1996). Peptide identity was checked by amino acid analysis, and by MALDI-TOF (Protein Service Labor, ETH Zürich).

The antibody fragment scFv(G3), specific for *S*-(2,4-dinitrophenyl)glutathione portion of the modified Glutathione-PEP, was isolated from the antibody phage library ETH-2 (a modified version of the synthetic antibody library reported by Pini *et al.*, 1998) by alternative panning with BSA (bovine serum albumin) or ovalbumin chemically coupled to *S*-(2,4-dinitrophenyl)glutathione according to standard techniques (Nissim *et al.*, 1994). Further details are available upon request from demartis@mol.biol.ethz.ch.

S-(2,4-Dinitrophenyl)glutathione

Six milligrams of 1-chloro-2,4-dinitrobenzene (3×10^{-2} mmol, solubilized in 200 µl of DMSO; Aldrich) and 6 mg of GSH (2×10^{-2} mmol, solubilised in 500 µl water and DMSO; Sigma) were reacted at room temperature for one hour in the presence of GST (0.01 mg/ml). The reaction product was HPLC-purified and characterized by MALDI-TOF and NMR analysis.

Chemical modification of glutathione-PEP cysteine with 1-chloro-2,4-dinitrobenzene

A total of 0.4 mg of 1-chloro-2,4-dinitrobenzene (2×10^{-3} mmol, solubilized in 70 µl DMSO; Aldrich) and 0.6 mg of glutathione-PEP (2×10^{-4} mmol, solubilised in 130 µl water) were reacted at room temperature for one hour in the presence of GST (0.01 mg/ml). The reaction product was HPLC-purified and characterized by MALDI-TOF.

Biotinylated 4-chloro-3,5-dinitrobenzoic acid derivative (4)

Seven milligrams of 4-chloro-3,5-dinitrobenzoic acid (2.8×10^{-2} mmol; Aldrich) and 55 mg of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (2.9×10^{-1} mmol; Aldrich) solubilised in 500 ml of $CHCl_3$ were reacted at room temperature for five minutes. Then 3 mg of Biotin-ε-aminocaproyl hydrazide (8×10^{-3}; solubilised in DMSO; Sigma) were added. After 90 minutes the reaction product was HPLC-purified and characterised by MALDI-TOF and by NMR.

Vectors

Phagemid pDN323 is a derivative of pHEN1 (Hoogenboom *et al.*, 1991), in which the gene coding for the *myc* tag has been replaced by the *Xenopus laevis* calmodulin gene (Neri *et al.*, 1995). It was obtained by PCR amplification (one minute at 94 °C, one minute at 55 °C and two minutes at 72 °C for 25 cycles; conditions as described by Marks *et al.* (1991)) of the calmodulin gene with oligos CAMBANO and CAMG3FOR (Table 4) and of the fd phage gene III with oligos CAMG3BACK and LMB2 using pHEN1 as a template (Hoogenboom *et al.*, 1991). These two genes were then assembled by PCR (Marks *et al.*, 1991) using oligos CAMBANO and LMB2 (one minute at 94 °C, one minute at 55 °C and two minutes at 72 °C for 25 cycles). The resulting gene, coding for a calmodulin-amber-gene III fusion, was subcloned in the *Not*I/*Eco*RI sites of pHEN1.

pSD4 is a derivative of pDN323, coding for a 16 amino acid residue linker between calmodulin and the polylinker (Figure 7). The modified calmodulin-gIII gene fusion was obtained by PCR amplification using primers LMB2 and CamLinkI3not, using pDN323 as template. The resulting PCR product was subcloned into the *Not*I/*Eco*RI sites of pCANTAB6.

For pSD3, the glutathione-*S*-transferase gene from *S. japonicum* was PCR amplified from vector pGEX-4T (Pharmacia Biotech, Piscataway, NJ, USA) with oligos GSTBANCO and GSTFONOT1, then subcloned into the *Nco*I/*Not*I sites of pDN323. The same insert yielded vector pFV7 when subcloned into the *Nco*I/*Not*I sites of pCANTAB6 (Vaughan *et al.*, 1996), and vector PSD8 when subcloned into the *Nco*I/*Not*I sites of pSD4.

pFV5, which allows the display of the FLAG linear peptide (Hopp *et al.*, 1988) on calmodulin-tagged phage,

was obtained by PCR amplification using primers LMB3 and FLAGNOT and pHEN1 as template. The resulting product was subcloned into the *Hin*dIII/*Not*I sites of pDN323.

For pSM5, the gene of the anti-lysozyme antibody fragment scFv(D1.3), obtained by *Nco*I/*Not*I digestion from pDN5 (Neri *et al.*, 1996), was subcloned into the *Nco*I/*Not*I sites of pDN323.

For pAH1, the catalytic domain of the insulin receptor tyrosine kinase domain (Hubbard *et al.*, 1994) was PCR amplified with oligos IRKBANCO and IRKFONOT, then subcloned into the *Nco*I/*Not*I sites of pDN323. The same insert, subcloned into the *Nco*I/*Not*I sites of pCANTAB6 (Vaughan *et al.*, 1996), yielded vector pAH2.

pAH5 is a bicistronic phagemid vector derivative of pSD4 (Figure 2). The mutations H64A, A73L and the deletion 75-83 (Strausberg *et al.*, 1995) were introduced into the subtilisin BPN' (*Bacillus amyloliquefaciens*) gene as follows. Two gene fragments were PCR amplified with oligo pairs SBTbanco/GnnH64Afo, and Gnbamut/Genenfonot, respectively. These two PCR fragments were assembled and amplified with oligos SBTbanco and Genenfonot. The resulting gene was cloned with *Nco*I/*Not*I sites into pSD4, to yield phagemid pSUBT. Using standard techniques, a ribosome binding site (rbs) preceding an ompA leader, the Pro peptide of subtilisin and two stop signals were then introduced at the unique *Hin*dIII site of pSUBT:

```
HindIII                    rbs          M  K  K  T          ompA
AAGCTTACGAATTTCTAGATAACGAGGGCAAAAAATGAAAAAGACA.................
 A  Q  A  S  G  K  S  N      Pro          V  A  H  A  Y  *  *
GCGCAGGCCTCTGGGAAATCAAAC..............GTAGCACATGCGTACTGATAA
BglII HindIII
GATCTAAGCTT
```

For PSM6, the catalytic domain of the *lck* tyrosine kinase domain was PCR amplified with oligos LCKFONOT and LCKBANCO, then subcloned into the *Nco*I/*Not*I sites of pDN323.

pFV67 allows the display of a GST-calmodulin-pIII fusion protein in which the amber codon is inserted between the calmodulin and the glutathione-*S*-transferase. The calmodulin coding sequence, preceded by the TAC amber codon, was obtained by PCR amplification of the calmodulin gene of pDN323 vector with oligos AmbCaMbaNo2 and CGfo. A PCR assembly with oligos AmbCaMbaNo2 and LMB2 long allowed its fusion with the gene III already amplified from the pDN323 vector with oligos CGba and LMB2 long. The resulting product was cloned in the *Not*I/*Eco*RI sites of pFV7.

pFV68 allows the display of a calmodulin-GST-pIII fusion protein on the surface of filamentous phage (Figure 7). The calmodulin coding sequence, PCR amplified from pDN323 with oligos CaMBaNco and GSTCamfo, and the *S. japonicum* GST gene, PCR amplified from vector pGEX4T/2 (Pharmacia Biotech) with oligos GSTCaMba and GSTfoNot1, were fused by PCR assembly (Marks *et al.*, 1991) performed with oligos CamBaNco and GSTfoNot1. The resulting product was cloned in the *Nco*I/*Not*I sites of pHEN1.

For pFV70, the human glutathione-*S*-transferase gene coding for the A1-1 isoform, was PCR amplified with oligos GST-A1hl-ba and GST-A1hl-foNot from a human

Table 4. List of oligonucleotides

CAMBANO	5′- AGT TCC GCC ATA GCG GCC GCT GAC CAA CTG ACA GAA GAG CAG -3′
CAMG3FOR	5′- CTT TCA ACA GTC TAC TTT GCT GTC ATC ATT TGT ACA AAC -3′
CAMG3BACK	5′- CAA ATG ATG ACA GCA AAG TAG ACT GTT GAA AGT TGT TTA GC -3′
LMB2	5′- GTA AAA CGA CGG CCA GT -3′
LMB3	5′- CAG GAA ACA GCT ATG AC -3′
FLAGNOT	5′- GAG TCA TTC TGC GGC CGC CTT GTC ATC GTC GTC CTT GTA GTC CTG CAG CTG CAC CTG GGC CAT GG -3′
IRKBANCO	5′- ATC GAC CCA TGG CCC AGG TGT CCT CTG TGT TTG TGC CGG ACG AGT GGG AGG TG -3′
IRKFONOT	5′- GAG TCA TTC TGC GGC CGC CTC CTC ACT CTC GGG AGC CTT GTT C -3′
fdseq1	5′- GAA TTT TCT GTA TGA GG -3′
Gnbamut	5′- CGG AAC TCA CGT TGC CGG CAC AGT TCT GGC TGT TGC GCC AAG CGC ATC ACT T -3′
SBT baNco	5′- GGT TCC ATG GCC GCG CAG TCC GTG CCT TAC G -3′
GenenfoNot	5′- CCA AGG TTG CGG CCG CCT GAG CTG CCG CCT GTA CGT T -3′
GnnfoH64A	5′- GTG CCG GCA ACG TGA GTT CCG GCA GAG TTG TTG TCT TGG AAA GGA -3′
CamLink13not	5′- TCC ATG ATA GCG GCC GCA GGT GGC GGT GGA AGT GGC GGT GGT GGC TCT GCT GAC CAA CTG ACA GAA GAG -3′
GSTBANCO	5′- AAT CGA CCC ATG GCC CAG GTC CAG ATG TCC CCT ATA CTA GGT TAT TGG -3′
GSTFONOT1	5′- GAG TCA TTC TGC GGC CGC GGA TCC ACG CGG AAC CAG ATC CG -3′
LCKFONOT	5′- ATG CAA TGA TGC GGC CGC AGG CTG AGG CTG GTA CTG GCC CTC -3′
LCKBANCO	5′- TCT ATA GCC ATG GCC CAG GTG CAG AAG CCC CAG AAG CCG TGG TGG -3′
AmbCambano2	5′- AGT TCC GCC ATA GCG GCC GCT TAG GCT GAC CAA CM ACA GAA GAG C -3′
Cgfo	5′- ACT TTC AAC AGT ACC CTT TGC TGT CAT CAT TTG TAC -3′
Cgba	5′- CAA ATG ATG ACA GCA AAG GGT ACT GTT GAA AGT TGT TTA GC -3′
Cambanco	5′- AAT CGA CCC ATG GCC CAG GTC CAG GCT GAC CAA CTG ACA GAA GAG CAG -3′
GSTCamfo	5′- CAT ACC TCC GCC AGA TCC GCC ACC CTT TGC TGT CAT CAT TTG TAC AAA -3′
GSTCamba	5′- GGT GGC GGA TCT GGC GGA GGT ATG TCC CCT ATA CTA GGT TAT TGG -3′
GST-A1hl-ba	5′- GCC CAG GTG CAG GCA GAG AAG CCC AAG CTC CAC TAC TTC AAT GC -3′
GST-A1hl-foNot	5′- GAG TCA TTC TGC GGC CGC GCC AGA ACC AAA CCT GAA AAT CTT CCT TGC TTC -3′
GST-A1hl-baSfi	5′- ACT ATG GTC GCC GCC CAG CCG GCC ATG GCC CAG GTG CAG GCA GAG AAG CC -3′
LMB2long	5′- GTA AAA CGA CGG CCA GTG AAT TC -3′

liver cDNA library (Human liver QUICK-Clone, Clontech). A further PCR amplification of the product with oligos GST-A1hl-baSfi and GST-A1hl-foNot was necessary to introduce an *Sfi*I restriction site. The resulting product was inserted in the *Sfi*I/*Not*I sites of pSD4 vector.

Phage ELISA

Phages were prepared according to standard procedures, and phage ELISA assays were performed essentially as described (Nissim *et al.*, 1994), but using a commercialy available horseradish peroxidase-labelled anti-M13 antibody (Cat. no. 27-9411-01; Pharmacia Biotech, Piscataway, NJ, USA) as detecting reagent. Calmodulin binding activity was assayed using streptavidin-coated plates (Cat. no. 1645 492; Boehringer Mannheim, Germany), which had been incubated for ten minutes at room temperature with 100 µl of a 10^{-7} M solution of biotin-labelled CAAARWKKAFIAVSAANRFKKIS peptide (Montigiani *et al.*, 1996) in PBS (phosphate-buffered saline; 50 mM phosphate (pH 7.2), 100 mM NaCl). The plates were then washed three times with PBS, blocked for 20 minutes with 2% (w/v) skimmed milk in PBS (2% MPBS), then washed three times with PBS and used for ELISA. Binding assays in the presence of Ca^{2+} were performed by adding to each well 30 µl of 10% skimmed milk in TBSC (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM $CaCl_2$) and 80 µl phage in TBSC (10^{12} t.u./ml). After 30 minutes incubation at room temperature, the plates were washed five times with TBSC and 0.1% (v/v) Tween-20, and five times with TBSC. To each well, horseradish peroxidase-labelled anti-M13 antibody (diluted 1:2000 in 2% milk dissolved in TBSC) was then added. After 20 minutes incubation, the plates were washed five times with TBSC and 0.1% Tween-20, and five times with TBSC. The plate-bound peroxidase was detected with the ready-to-use BM-Blue soluble substrate (Cat. no. 1484281; Boehringer Mannheim, Germany). Binding in the absence of Ca^{2+} was tested in a similar fashion, but replacing TBSC with TBSE (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 20 mM EDTA) in all the steps.

For the other ELISA assays, antigens were coated on microtitre plates (Cat. no. Falcon 3912, Becton Dickinson Labware, Oxnard, CA, USA) overnight at room temperature as follows: anti-FLAG M2 antibody (Kodak), 5 µg/ml; hen egg lysozyme (Sigma, St. Louis, MO, USA), 3 mg/ml; anti-GST monoclonal antibody GST-2 ascites (Cat. no. G-1160; Sigma), 1:1000 dilution; anti-*lck* polyclonal antibody (2102, Cat. no. sc-13; Santa Cruz. Biotechnology, Inc., Santa Cruz, CA, USA), 5 µg/ml. PBS was used as buffer for the incubation and wash steps as described (Nissim *et al.*, 1994).

Stability of calmodulin-tagged phage-peptide complexes

A 10^{-8} M solution of a biotinylated calmodulin-binding peptide (CAAARWKKAFIAVSAANRFKKIS or CAAARAKKNFIAVSAANRFKKIS; Montigiani *et al.*, 1996) was added to calmodulin-tagged phage preparations (10^{12} t.u./ml) in TBSC and incubated for ten minutes. To the resulting mixture, aliquoted in different tubes, unlabelled CAAARWKKAFIAVSAANRFKKIS peptide in 1000-fold molar excess (10^{-5} M) was added at different times. The fraction of phage particles in which the biotinylated peptide had been replaced by the unlabelled peptide was then determined by ELISA as described in the previous section.

Stability of calmodulin-tagged phage peptide complexes to PEG precipitation was checked as follows. A 10^{-8} M solution (final concentration) of a biotinylated calmodulin-binding peptide CAAARWKKAFIAVSAANRFKKIS was added to 1 ml of calmodulin-tagged phage (10^{12} t.u./ml) in TBSC and incubated for ten minutes. To this solution, 20% (v/v) polyethyleneglycol 6000 and 2.5 M NaCl was added (200 µl) and the resulting mixture was incubated on ice for one hour, then centrifuged in a bench centrifuge (13,000 rpm for five minutes). The pellet was resuspended in 1 ml of TBSC. The titres of the phage solutions before and after PEG precipitation were then determined. The fraction of phage particles that were still peptide-bound after the PEG precipitation procedure was evaluated by ELISA, using a streptavidin-coated plate (see previous section).

Rescue of calmodulin-tagged enzymes on phage

Phage displaying enzyme-calmodulin as gene III-product fusion (GST-CaM from vector pSD3; insulin receptor tyrosine kinase-CaM from pAH1) was mixed with a molar excess of phage displaying only the enzyme as gene III-product fusion (from vectors pFV7 and pAH2, respectively; see previous section) in 1 ml of TBSC containing 2% BSA and 10^{-9} M biotin-CAAARWKKAFIAVSAANRFKKIS peptide. After ten minutes incubation, 100 µl of streptavidin-coated M280 Dynabeads (Cat. no. 112.05; 10 mg beads/ml; DYNAL, Oslo, Norway), preblocked in TBSC containing 2% BSA, was added. The tubes were mixed for ten minutes, and the magnetic beads were captured on a magnet (Cat. no. 120.04; DYNAL) and washed five times with TBSC and 0.1% Tween-20, then five times with TBSC. Phages were eluted with TBSE (five minutes incubation), saturated with calcium, used to infect exponentially growing TG1 *E. coli* cells (Gibson, 1984), then plated on TYE agar plates containing 100 mg/l ampicillin and 1% (w/v) glucose. Phage for further rounds of panning was produced as described (Nissim *et al.*, 1994). The ratio of the two phages after selection was obtained by PCR screening of single colonies (Marks *et al.*, 1991), using oligos LMB3 and fdseq1 (Figure 2(a)).

Submut-CaM-phage (10^{10} t.u.; obtained from phagemid pAH5), was incubated at room temperature for five minutes in TBSC, in the presence or the absence of 100 nM DYKDEGGGAAARWKKAFIAVSAANRFKKIS peptide. The resulting peptide phage complex was precipitated in PEG (as described above) resuspended in 100 µl of TBSC. Streptavidin-coated M280 Dynabeads (50 µl) were diluted to 500 µl in 3% BSA/TBSC, containing 0.4 µg of biotinylated M1 anti-FLAG antibody (Kodak). Unbound antibody was removed by magnetic capture; the resulting beads were resuspended in 100 µl of 3% BSA/TBSC and incubated with the phage-peptide complex for 30 minutes. Subsequent washing, elution and titre determination were then performed.

Capture experiments with the *in situ* generation of reaction products were performed at substrate concentrations comparable to the K_M of the enzyme. Submut: 1 g/l genenase I (New England Biolabs; kind gift from Dr Paul Carter, Genentech Inc., San Francisco CA) was incubated in the presence or in the absence of 0.4 g/l GAAHYDYKDEGGGAAARWKKAFIAVSAANRFKKIS peptide in 5 µl of buffer S (0.5 mM DTT, 500 mM NaCl, 50 mM Tris-Cl (pH 8), 5 mM $CaCl_2$, 50 mg/l PMSF) for

45 minutes at 37 °C. To these mixtures, 75 µl of TBSC and 20 µl of CaM-phage (10^{11} t.u., from pSD4) were added, followed by incubation at room temperature for 15 minutes. The subsequent capture with M1-streptavidin-Dynabeads was performed as described above.

Glutathione-PEP (12 µl; 10^{-3} M) and 10 µl of 1-chloro-2,4-dinitrobenzene (5×10^{-3} M) were allowed to react for five minutes at room temperature in the presence of GST (0.08 mg/ml). To this reaction mixture, calmodulin-phage (pSD4; 10^{11} t.u.) and TBSC were added, to a final volume of 100 µl. After five minutes incubation, the mixture was precipitated twice in PEG, resuspended in 100 µl of TBSC containing 3% BSA, and captured onto scFv(G3)-coated Dynabeads, which were prepared as follows.

Streptavidin-coated M280 Dynabeads (50 µl), preblocked in TBSC containing 3% BSA, were reacted with biotinylated antibody M2 (M2 reacts with the FLAG peptide tag of the scFv, Pini *et al.*, 1998) The tubes were mixed for ten minutes, then the magnetic beads were captured on a magnet and washed twice with TBSC. The beads were then resuspended in TBSC containing 3% BSA and 5 µg/ml of purified scFv(G3) After 30 minutes mixing, the magnetic beads were captured on the magnet, and washed three times with TBSC.

Phage captured onto scFv(G3) Dynabeads were washed five times with TBSC and 0.1% Tween-20, three times with TBSC, and eluted with TBSE (five minutes incubation). The resulting solution was saturated with calcium chloride, used to infect exponentially growing TG1 *E. coli* cells, then plated on TYE agar plates containing 100 mg/l ampicillin and 1% glucose.

Phage titre determinations

Phage preparations with the cleavable helper phage KM13 were obtained and titered as described (Kristensen & Winter, 1998). Briefly, phage particles produced with KM13 or VCS-M13 helper phages were either titered directly or incubated 15 minutes at 37 °C after addition of one-tenth in volume of bovine pancreatic trypsin (10 g/l, Sigma). A molar excess of *E. coli* TG1 in 2 × TY at a density of about 4×10^8 cells/ml was then added to the phage and incubated at 37 °C for 30 minutes. The cells were plated on ampicillin-containing plates and grown overnight at 37 °C.

Selections with the submut-calmodulin-phage

Submut-CaM-phage (10^{10} t.u.) was incubated in 200 µl of buffer Z (0.5 mM DTT, 200 mM NaCl, 50 mM Tris-HCl (pH 8), 1 mM $CaCl_2$, 50 mg/l PMSF) with 10 mg/l peptide DYKDEGGGAAARWKKAFIAVSAANRFKKIS, GAAHYDYKDEGGGAAARWKKAFIAVSAANRFKKIS or GDDDDKDYKDEGGGAAARWKKAFIAVSAANRFKKIS at 37 °C for 15 minutes. After precipitation with PEG, phages were captured with M1-streptavidin-Dynabeads as described above.

In a competition experiment, submut-CaM-phage (9×10^9 t.u.) was mixed with GST-CaM-phage (10^{10} t.u.) and 10 mg/l peptide (either GAAHYDYKDEGGGAAARWKKAFIAVSAANRFKKIS or GDDDDKDYKDEGGGAAARWKKAFIAVSAANRFKKIS) in 200 µl of buffer Z (above). A small aliquot of this mixture was used to infect TG1 bacteria. PCR screening of the resulting colonies was used to confirm the ratio of phages used for selection. After 15 minutes at 37 °C and one PEG-precipitation, phages were captured with M1-streptavidin-Dynabeads as described above, and used to infect exponentially growing TG1 bacteria. The ratio of the two phages after selection was obtained by PCR screening of single colonies using oligos LMB3 and fdseq1.

Selections with GST-calmodulin-phage

GST-CaM-phage (pSD8; 10^{10} t.u.), preblocked in TBSC containing BSA (10 mg/ml), were added to a 10^{-7} M glutathione-PEP solution in 180 µl TBSC. After 30 seconds incubation, 20 µl of 5 mM biotinylated 4-chloro-3,5-dinitrobenzoic acid derivative ((4) Figure 8) was added. After 15 seconds of incubation, the reaction mix was precipitated twice in PEG, resuspended in 200 µl of TBSC containing 3% (w/v) milk powder and BSA (1 mg/ml), captured on streptavidin-coated Dynabeads and eluted with TBSE as described above.

The reactivity of the thiol group of glutathione-PEP on phage was assayed as follows. GST-CaM-phage (pSD8; 10^{10} t.u.), preblocked in TBSC containing BSA (10 mg/ml), was added to 180 µl of TBSC solution, containing either 0.1 µM glutathione-PEP, biotin-CAAARWKKAFIAVSAANRFKKIS, or no peptide. After 30 seconds incubation, 20 µl of 5 mM Biotin-HPDP (Pierce) was added. After 15 seconds, the reaction mixture was precipitated with PEG twice and captured on streptavidin-coated Dynabeads as described above. Phages were eluted by five minutes incubation either with TBSE or with a 20 mM DTT solution, used to infect exponentially growing TG1 *E. coli* cells, then plated on TYE. agar plates containing 100 mg/l ampicillin and 1% glucose.

Measurements of enzymatic parameters and calculations of the time requirements for catalysed and uncatalysed reaction of substrates anchored on phage.

The kinetic parameters (k_{cat}, k_{uncat}, K_M) of the GST-catalysed reaction between 4-chloro-3,5-dinitrobenzoic acid and glutathione in PBS (pH 7.2) at 25 °C, were measured spectrophotometrically as described (Habig *et al.*, 1974).

In the reaction scheme of Figure 8, the effective concentration of glutathione anchored on phage can be assumed to be grater than the K_M of GST for glutathione. At concentrations of biotinylated 4-chloro-3,5-dinitrobenzoic acid derivative (4) greater than K_M, the time required for one GST-catalysed reaction on phage is approximately equal to $1/k_{cat}$.

The uncatalysed reaction of phage-anchored glutathione with a molar excess of (4) follows a pseudo-first order reaction kinetic (Fersht, 1984). We can therefore calculate that the time necessary to react 0.01% of phage-anchored glutathione with 4-chloro-3,5-dinitrobenzoic acid derivatives (4) at a concentration [4] is equal to:

$$T_{0.01\%} = [-\ln(0.9999)]/(k_{uncat} \times [4])$$

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